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Necrostatin-1 supplementation enhances young porcine islet maturation and in vitro function

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Abstract

Background: Necroptosis has been demonstrated to be a primary mechanism of islet cell death. This study evaluated whether the supplementation of necrostatin-1 (Nec-1), a potent inhibitor of necroptosis, to islet culture media could improve the recovery, maturation, and function of pre-weaned porcine islets (PPIs).

Methods: PPIs were isolated from pre-weaned Yorkshire piglets (8-15 days old) and either cultured in control islet culture media (n = 6) or supplemented with Nec-1 (100 μM, n = 5). On days 3 and 7 of culture, islets were assessed for recovery, insulin content, viability, cellular composition, GLUT2 expression in beta cells, differentiation of pancreatic endocrine progenitor cells, function, and oxygen consumption rate.

Results: Nec-1 supplementation induced a 2-fold increase in the insulin content of PPIs on day 7 of culture. When compared to untreated islets, Nec-1 treatment doubled the beta- and alpha-cell composition and accelerated the development of delta cells. Additionally, beta cells of Nec-1-treated islets had a significant upregulation in GLUT2 expression. The enhanced development of major endocrine cells and GLUT2 expression after Nec-1 treatment subsequently led to a significant increase in the amount of insulin secreted in response to in vitro glucose challenge. Islet recovery, viability, and oxygen consumption rate were unaffected by Nec-1.

Conclusion: This study underlines the importance of necroptosis in islet cell death after isolation and demonstrates the novel effects of Nec-1 to increase islet insulin content, enhance pancreatic endocrine cell development, facilitate GLUT2 upregulation in beta cells, and augment insulin secretion. Nec-1 supplementation to culture media significantly improves islet quality prior to xenotransplantation.

KEYWORDS

diabetes, islet development, islet transplantation, necrostatin-1, porcine islets

1 | INTRODUCTION

The advancement in islet transplantation offers a promising cure for patients with type 1 diabetes, especially with the improved islet isolation procedures and the use of a glucocorticoid-free immunosuppressive protocol.[1-3] Despite these successful outcomes, the large-scale clinical application of islet transplantation has been limited by a shortage of donor pancreata and the lack of quality islets after islet isolation.[4,5] With the recent progress in gene editing technology and islet xenotransplantation, the use of pancreata from young porcine donors has the potential to serve as an unlimited alternative source of islets.[6-10]

The current clinical islet xenotransplantation protocol required porcine islets to undergo prolonged culture after isolation due to their immaturity.[11] Young porcine islets from neonatal (1-3 days old) and pre-weaned pigs (4-11 days old) are commonly cultured for 5-10 days prior to transplantation.[12-15] A study has demonstrated that 12-day culture of young porcine islets improved the rates of diabetes reversal, yet another study has shown that these islets required to be cultured for 21 days for optimal maturation.[16,17] Although pre-transplant culture of islets is beneficial to improve the outcomes of islet xenotransplantation, substantial islet loss during this culture period has been reported in young porcine islets.[17,18]

While apoptosis is considered as a primary regulated cell death pathway, recent studies have identified several other pathways of regulated cell deaths, including apoptosis, necroptosis, ferroptosis, pyroptosis, and parthanatos.[19] Necroptosis is a form of regulated necrosis that has recently been suggested to be a major pathway of islet cell death in the development of diabetes and during both in vitro culture and islet transplantation.[19,20] The inflammatory cascade triggered by the release of danger-associated molecular patterns (DAMPs), such as high-mobility group box 1 (HMGB1) and DNA fragments, after islet necroptosis furtherly contributes to islet damage, reduced islet survival, enhanced immune responses after implantation, and consequently impaired outcomes of islet transplantation in both mice and humans.[21-23]

Necrostatin-1 (Nec-1) can block necroptosis by acting as a specific allosteric inhibitor of receptor-interacting protein-1 (RIP1) kinase, a downstream signaling molecule of the death receptor–induced non-apoptotic cell death pathways.[24] The anti-necroptotic activity of Nec-1 has been demonstrated in multiple cell types, including Jurkat, BALB/c 3T3, and U937 cells, and in an in vivo model of ischemic brain injury.[25] Pretreatment of Nec-1 in a rat model of retinal ischemia-reperfusion injury significantly reduced neuronal cell death and neuronal degeneration of the inner retina, leading to improved functional outcomes.[26] In a mouse model of myocardial ischemia, Nec-1 treatment at the time of reperfusion conferred cardioprotective effect by reducing cell death and infarct size.[27,28]

In the past decade, the effect of Nec-1 and its downstream effect to inhibit necroptosis in islet cells have not been fully elucidated. In a study utilizing both beta-cell lines and isolated mouse islets, Nec-1 inhibited cell death after nitric oxide exposure.[23] The study further showed that the nitric oxide–induced release of the DAMPs, HMGB1 and cyclophilin A, was significantly reduced after Nec-1 treatment in cultured βTC-6 and INS-1 cells.[23] After encapsulated human islets were cultured for 7 days in either normal or low-nutrient condition at 1% of oxygen, a significant decrease in the amount of nuclear DNA content was observed in control islets, but not islets treated with Nec-1.[29] Nec-1 treatment also reduced the release of the DAMPs, dsDNA and uric acid, in encapsulated human islets after 1- and 7-day culture in low-nutrient and hypoxic conditions.[29] A limitation of these studies in identifying the effect of Nec-1 on islet cells is that only the release of DAMPs into the extracellular environment and cell survival were assessed after Nec-1 treatment, but no other islet quality measurements were done, such as islet composition and function.[30] Furthermore, whether young porcine islets, a viable alternative source of islets for transplantation, are responsive to Nec-1 treatment during culture has never been studied.

With the possibility that Nec-1 could maintain porcine islet mass and augment islet quality before islet transplantation, the present study examined the effect of Nec-1 on the survival, maturation, and insulin secretory function of pre-weaned porcine islets (PPIs) during in vitro maturation culture.

2 | MATERIALS AND METHODS

2.1 | PPI isolation

All animal procedures were performed under approved University of California Irvine, Institutional Animal Care and Use Committee. PPIs were isolated from the pancreata of 8- to 15-day-old, pre-weaned Yorkshire pigs as previously described.[14] In brief, pancreata were procured (≤10 minutes) while the donor was under anesthesia and stored in cold HBSS. Cold ischemic time was limited to less than 1 hour. Pancreata were finely minced into 1 mm^3 pieces and digested with Sigma Type V Collagenase (2.5 mg/mL, dissolved in HBSS; cat# C8051, Sigma-Aldrich) in 37°C, 100 rpm shaking water bath for 15 minutes. The digestion was quenched using HBSS supplemented with 1% porcine serum (cat# 26250084, Gibco-Thermo Fisher Scientific). Digested pancreatic tissues were filtered through 500 μm metal mesh.

2.2 | Islet culture and Nec-1 treatment

PPIs were cultured in islet maturation culture media, composed of Ham's F-12 medium (Corning Inc, cat#10-080), HEPES (Sigma-Aldrich, cat#H3375), L-glutathione (Sigma-Aldrich, cat#G4251), ITS+3 (Sigma-Aldrich, cat#I2771), nicotinamide (Sigma-Aldrich, cat#N5535), gentamycin sulfate (Corning Inc, cat#30-005-CR), Trolox (Sigma-Aldrich, cat#238813), heparin (Sagent Pharmaceuticals, cat#400-10), Pefabloc (Santa Cruz Biotechnology, cat#sc-202041B), L-glutamine (Alfa Aesar, cat#56-85-9), medium 199 (Corning Inc, cat#50-051-PB), calcium chloride dihydrate (Fisher Scientific, cat#C79-3), DNase (Sigma-Aldrich, cat#D4263), antibiotic/antimycotic solution (Corning Inc, cat#30-004-CI), and 10% porcine serum.[12] After islet isolation, the islets were divided into 2 groups to be cultured in islet maturation culture media alone (n = 5) or supplemented with Nec-1 (100 μM, Abcam, cat#ab141053, n = 5).[29] All islets were cultured for 7 days in T-150 untreated suspension flasks (cat # CLS430825, Corning Inc) in a 37°C, 5% CO2 humidified incubator (cat#3110, Thermo Forma Series II 3120 Water Jacketed CO2 Incubators). 100% media change was performed on day 1, and 50% media change was performed on days 3 and 5. Islets were collected on days 3 and 7 for assessment.

2.3 | Islet assessment

2.3.1 | Islet recovery

Islet equivalence (IEQ) was determined by collecting an aliquot of 100 μL for staining with 1mL dithizone (DTZ, MP Biomedicals, cat#150999) for 5 minutes.[14] Stained islets were counted using a standard stereomicroscope (Max Erb) with a 10x eyepiece graticule.[31] Islet recovery was expressed as the percentage of IEQ per gram of pancreatic tissue normalized to day 3 of the control group.[32]

2.3.2 | Islet viability

100 IEQ was stained with Calcein AM (CalAM, Invitrogen, cat#C1430) for live cells and propidium iodide (PI, Invitrogen, cat#P3566) for dead and dying cells for 30 minutes. Stained islets were analyzed using a microplate reader (Tecan Infinite F200; Tecan). The islet viability was calculated by the equation: CalAM-positive cells/(CalAM-positive cells+PI-positive cells) × 100.

2.3.3 | Islet cellular viability, composition, GLUT2 expression, and differentiation

3000 IEQ was dissociated using Accutase (cat# AT104-500, Innovative Cell Technologies) for 15 minutes in a 37°C, 100 rpm shaking water bath and filtered through a 40-μm filter (VMR) to obtain a single-cell suspension.[30] Cell samples were stained on ice for 30 minutes to detect live and dead cells using 7-aminoactinomycin D dye (7-AAD; cat#A1310, Invitrogen). Stained cells were fixed with 4% paraformaldehyde for 10 minutes and permeabilized using Intracellular Staining Permeabilization Wash Buffer (cat#421002, BioLegend 1) for 15 minutes on ice. After washing, cells were incubated in Protein Block (cat# ab64226, Abcam) for 30 minutes on ice to reduce non-specific binding and followed by staining with fluorescently conjugated antibodies in Intracellular Staining Permeabilization Wash Buffer (cat#421002, BioLegend) with 0.5% bovine serum albumin (BSA; cat#BAL62-0500, Equitech-Bio, Inc) for 30 minutes on ice. PE-conjugated anti-insulin (cat#8508, CST), APC-conjugated anti-glucagon (cat#NBP2-21803AF647, Novus Biological), and PE-conjugated anti-somatostatin (cat# NBP2-37447PE, Novus Biological) antibodies were used as a marker for beta cells, alpha cells, and delta cells, respectively. FITC-conjugated anti-GLUT2 (cat#FAB1414G-100UG, Novus Biological) antibody was used for double staining with PE-conjugated anti-insulin (cat#8508, CST) to identify GLUT2-positive beta cells. FITC-conjugated anti-neurogenin 3 (cat#bs-0922R, Bioss) and APC-conjugated anti-Nkx6.1 (cat#563338, BD Pharmingen) were used as markers for pancreatic endocrine progenitor cells. Stained cells were quantified using the NovoCyte 3000VYB Flow Cytometer (ACEA Biosciences, Inc). Cell populations were then analyzed using FlowJo software (FlowJo). An unstained, single-stained, fluorescence minus one, and matching isotype control were used as gating controls.

2.3.4 | Islet function

In vitro function of PPIs was determined by glucose-stimulated insulin release (GSIR) assay.[14] A triplicate of 100 IEQ per sample was incubated at 37°C and 5% CO2 for 1 hour in each media in the corresponding order: low glucose (2.8 mM; L1), high glucose (28 mM; H), low glucose (2.8 mM; L2), and high glucose plus 3-isobutyl-1-methylxanthine (28 mM + 0.1 mM IBMX; H+). Supernatant was collected and stored at −20°C until analysis. Insulin concentration was evaluated using a standard porcine insulin enzyme-linked immunosorbent assay (Porcine Insulin ELISA; cat# 10-1200-01, Mercodia) and calculated using a microplate reader (Infinite F200, Tecan and Magellan V7). The secreted insulin was normalized to the DNA content as described below in each sample and expressed as pg of insulin/ng of DNA. The stimulation index (SI) was obtained from dividing the insulin secreted in the high glucose media over the insulin secreted in the first low glucose media.

2.3.5 | Oxygen consumption rate (OCR)

The OCR of islets from each sample was determined by loading 200 IEQ into a capped titanium chamber connected to a fiber optic sensor filled with 37°C serum-free RPMI-1640 media (cat# FOL/C2T175P, Instech Laboratories). As previously described, the OCR was calculated as the linear decrease in the partial pressure of oxygen inside the chamber with time.[33] The oxygen sensor was calibrated at zero percent oxygen and oxygen-saturated serum-free RPMI-1640 media before islet measurements. The OCR was normalized to the DNA content in each sample and expressed as Nmol/min·mg DNA.

2.3.6 | Islet insulin content

150 IEQ was collected on day 3 and day 7 of culture and lysed with cell lysis buffer (10 mM Tris-HCl, 1 mM EDTA, 1% Triton X-100, pH 8). Islet cells were sonicated (Sonics VibraCell Ultrasonic Processor Model VC70T, Sonics & Materials, Inc) on ice for 30 seconds to ensure complete cell lysis. The sample was centrifuged at 1400 *g* for 15 minutes at 4°C. Supernatant was quantified for insulin content using a standard porcine insulin enzyme-linked immunosorbent assay (Porcine Insulin ELISA; cat# 10-1200-01, Mercodia) and calculated using a microplate reader (Infinite F200, Tecan and Magellan V7).[34] Insulin content was normalized to the sample DNA content and expressed as pg of insulin/ng of DNA.

2.3.7 | Islet DNA content

Islets from culture, GSIR, and OCR assays were collected and lysed with cell lysis buffer (10 mM Tris-HCl, 1 mM EDTA, 1% Triton X-100, pH 8). Islet cells were sonicated (Sonics VibraCell Ultrasonic Processor Model VC70T, Sonics & Materials, Inc) on ice for 30 seconds to ensure complete cell lysis. After sonication, the sample was centrifuged at 1400 *g* for 15 minutes at 4°C and the supernatant was collected. The DNA content from the collected supernatant was quantified with a

fluorescent DNA stain (Quant-iT PicoGreen dsDNA kit; cat #Q32850, Molecular Probes) and measured using a microplate reader (Infinite F200, Tecan and Magellan V7).[34]

2.4 | Statistical analysis

All data are expressed as mean ± standard error of mean (SEM). A one-way ANOVA followed by a post hoc Tukey's HSD test was performed to determine statistical significance. *P*-values < .05 were considered to be statistically significant. Data were analyzed using GraphPad Prism (GraphPad Software 8.0.1).

3 | RESULTS

3.1 | The effect of Nec-1 supplementation to islet culture media on islet recovery

Nec-1 was added to our standard tissue islet culture media after islet isolation to examine its effect on pre-weaned porcine islets. The islet recovery was calculated by normalizing all islet counts (IEQ/g of pancreatic tissue) to the islet count on day 3 of the untreated group. Islets treated with Nec-1 (95.60 ± 11.79% of control, mean ± SEM) had no significant difference in the number of islets recovered on day 3 of culture compared to untreated islets (*P* = NS, Figure 1). Islet loss was significantly higher in both the untreated (51.49 ± 7.81% of control) and Nec-1 treated islets on day 7 (45.72 ± 9.01% of control) compared to untreated islets on day 3 of culture (*P* < .01, Figure 1). Similar to the decrease in untreated islets with culture time, Nec-1-treated islets had a significant decrease in the number of islets on day 7 compared to Nec-1-treated islets on day 3 of culture (*P* < .01, Figure 1).

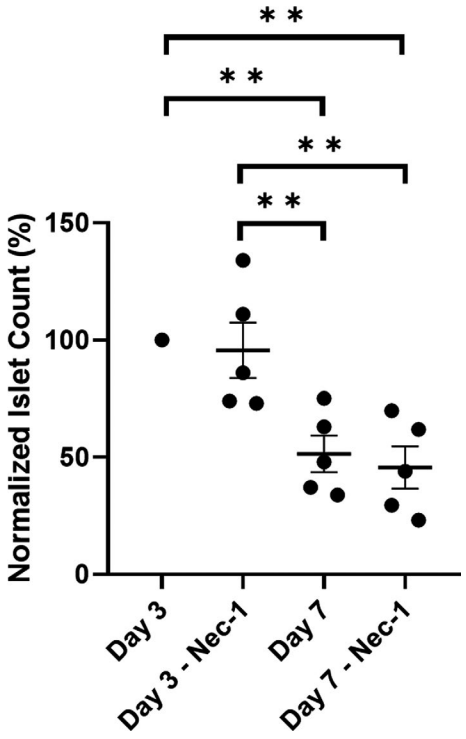

FIGURE 1 Islet recovery on days 3 and 7 of culture in control media or media supplemented with Nec-1 on day 0 of culture. Islet recovery was calculated as the percentage of IEQ per gram of pancreatic tissue normalized to day 3 of untreated islets. n = 5 for each group. *P < .05. **P < .01. Data expressed as mean ± SEM

3.2 | The effect of Nec-1 supplementation to islet culture media on islet viability

The islet viability was determined by Calcein AM and propidium iodide staining on days 3 and 7 of culture. There was no significant difference in the viability of untreated islets on day 3 (95.60 ± 1.21%) and day 7 of culture (93.40 ± 1.54%) compared to Nec-1-treated islets on day 3 (92.40 ± 0.75%) and day 7 of culture (97.60 ± 0.40%) (*P* = NS, Figure 2).

3.3 | The effect of Nec-1 supplementation to islet culture media on islet insulin content

On day 3 of culture, Nec-1-treated islets (56.11 ± 15.64 pg insulin/ng DNA) had no significant difference in the insulin content when compared to untreated islets on both day 3 (34.89 ± 5.27 pg insulin/ng DNA) and day 7 of culture (40.26 ± 7.12 pg insulin/ng DNA) (*P* = NS, Figure 3). After 7 days of culture, islets treated with Nec-1 (93.15 ± 17.25 pg insulin/ng DNA) had a 2-fold increase in the insulin content compared to untreated islets on days 3 and 7 of culture (*P* < .05, Figure 3). The insulin content of Nec-1-treated islets was not significantly different on days 3 and 7 of culture (*P* = NS, Figure 3).

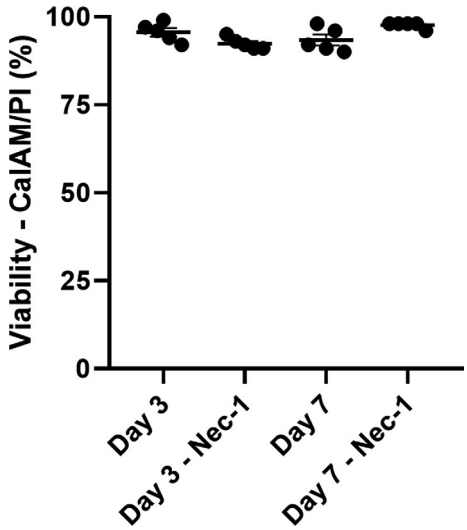

FIGURE 2 Islet viability on days 3 and 7 of culture in control media or media supplemented with Nec-1 on day 0 of culture. 100 IEQ were stained with Calcein AM (CalAM) for live cells and propidium iodide (PI) for dead and dying cells for 30 minutes on days 3 and 7 of culture. The islet viability was calculated by the equation: CalAM-positive cells/(CalAM-positive cells + PI-positive cells) × 100. n = 5 for each group. Data expressed as mean ± SEM

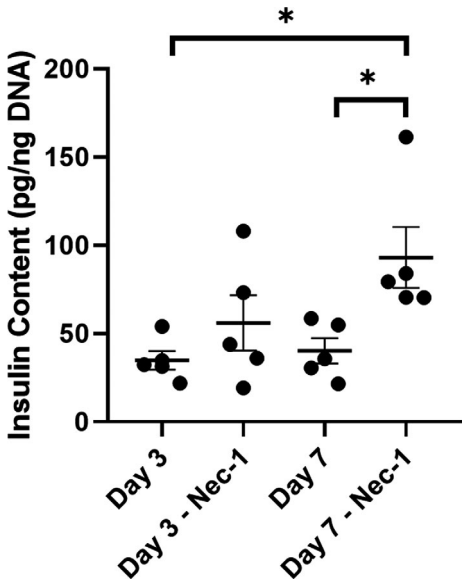

FIGURE 3 Insulin content of PPIs on days 3 and 7 of culture in control media or media supplemented with Nec-1 on day 0 of culture. 150 IEQ per isolation was lysed, sonicated, and evaluated for insulin content on days 3 and 7 using standard porcine insulin ELISA. The amount of insulin was normalized to the sample DNA content. n = 5 for each group. *P < .05. Data expressed as mean ± SEM

3.4 | The effect of Nec-1 supplementation to islet culture media on cellular viability, composition, and GLUT2 expression in beta cells

After dissociation, the viability of untreated and Nec-1-treated islet cells was similar throughout 7-day culture. On days 3 and 7 of culture, 90.10 ± 1.75% and 88.66 ± 2.87% of untreated islet cells were viable compared to 92.98 ± 1.62% and 91.16 ± 1.71% of Nec-1-treated islet cells (P = NS, Figure 4A).

When compared to untreated islets on day 3 of culture (4.86 ± 0.27%), a 2-fold increase in the beta-cell composition of Nec-1-treated islets (10.98 ± 1.81%) on the same day of culture was observed (P < .05, Figure 4B). Nec-1 treatment resulted in a 3-fold increase in the beta-cell composition on day 7 (16.14 ± 1.66%) compared to untreated islets on day 3 of culture (P < .01, Figure 4B). Nec-1 treatment doubled the level of beta cells in comparison with untreated islets on day 7 of culture (9.15 ± 1.59%; P < .05, Figure 4B).

Similarly, islets treated with Nec-1 had a 3-fold and 5-fold increase in alpha-cell composition on day 3 (8.72 ± 1.32%) and day 7 (15.82 ± 3.09%), respectively, when compared to untreated islets on day 3 (1.96 ± 0.33%) of culture (P < .05, Figure 4C). The percentage of alpha cells in Nec-1-treated islets was 2-fold higher than untreated islets (6.69 ± 0.58%) on day 7 of culture (P < .01, Figure 4C). In addition, Nec-1 islets had a significant increase in the level of alpha cells from day 3 to day 7 (P < .05, Figure 4C). However, this was not observed in the untreated group (P = NS, Figure 4C).

The 2-fold increase in the delta-cell composition of Nec-1-treated islets after 3 days of culture (2.47 ± 0.19%) with respect to untreated islets on day 3 (1.12 ± 0.16%) was comparable to untreated islets on day 7 (2.49 ± 0.29%) (P < .01, Figure 4D). The level of delta cells on day 7 of Nec-1-treated islets (2.36 ± 0.24%) was also significantly higher than untreated islets on day 3 of culture (P < .05, Figure 4D). However, there was no significant difference between the Nec-1-treated and untreated groups on day 7 (P = NS, Figure 4D).

In comparison with untreated islets on day 3 of culture (16.74 ± 5.95%), Nec-1-treated islets on day 3 (51.18 ± 7.43%) and day 7 of culture (48.60 ± 8.95%) had a 2-fold increase in the expression of GLUT2 in beta cells (P < .05, Figure 4E). While the percentage of GLUT2-positive beta cells was higher in Nec-1-treated islets on day 3 and day 7 compared to untreated islets on day 7 of culture (29.85 ± 3.22%), there was no statistically significant (P = NS, Figure 4E).

3.5 | The effect of Nec-1 supplementation to islet culture media on the differentiation of pancreatic endocrine progenitor cells

Ngn3 is a key transcription factor in the differentiation of pancreatic precursor cells to endocrine progenitor cells.[35] Even though the number of Ngn3-positive endocrine progenitor cells markedly decreased with culture time in both untreated islets (day 3 = 80.50 ± 7.43% and day 7 = 61.20 ± 3.68%) and islets treated with Nec-1 (day 3 = 58.82 ± 16.27% and day 7 = 40.16 ± 4.89%), no statistical significant difference was observed between these islets on day 3 and day 7 of culture (P = NS, Figure 5A).

The transcription regulator Nkx6.1 is essential for the final development of endocrine progenitor cells to fully developed beta cells.[35] Prolonged culture improved beta-cell differentiation in both untreated islets (day 3 = 7.01 ± 1.44% and day 7 = 15.86 ± 0.70%) and islets treated with Nec-1 (day 3 = 2.43 ± 0.24% and day 7 = 8.47 ± 1.25%) as observed by the significant increase in the percentage of Nkx6.1-positive endocrine progenitor cells (P < .01, Figure 5B). When compared to untreated islets on both day 3 and day 7 of culture, Nec-1-treated islets had a significantly lowered number of Nkx6.1-positive endocrine progenitor cells (P < .01, Figure 5B).

3.6 | The effect of Nec-1 supplementation to islet culture media on islet function

Glucose-stimulated insulin release assay was used to evaluate islet function. Untreated and Nec-1-treated islets were incubated for 1 hour in each media in the corresponding order: first low glucose (2.8 mM; L1), high glucose (28 mM; H), second low glucose (2.8 mM; L2), and high glucose plus 3-isobutyl-1-methylxanthine (28 mM + 0.1 mM IBMX; H+). The amount of secreted insulin per hour in each glucose media was normalized to the DNA concentration. Nec-1-treated islets on day 3 of culture (L1 = 1.56 ± 0.21 pg/ng DNA/h, H = 2.79 ± 0.22 pg/ng DNA/h, L2 = 1.40 ± 0.20 pg/ng DNA/h, H+ = 5.11 ± 0.83 pg/ng DNA/h) had a 4-fold increase in the insulin secretion in L1, H, L2, and H+ glucose media in comparison with untreated islets assessed on the same day of culture

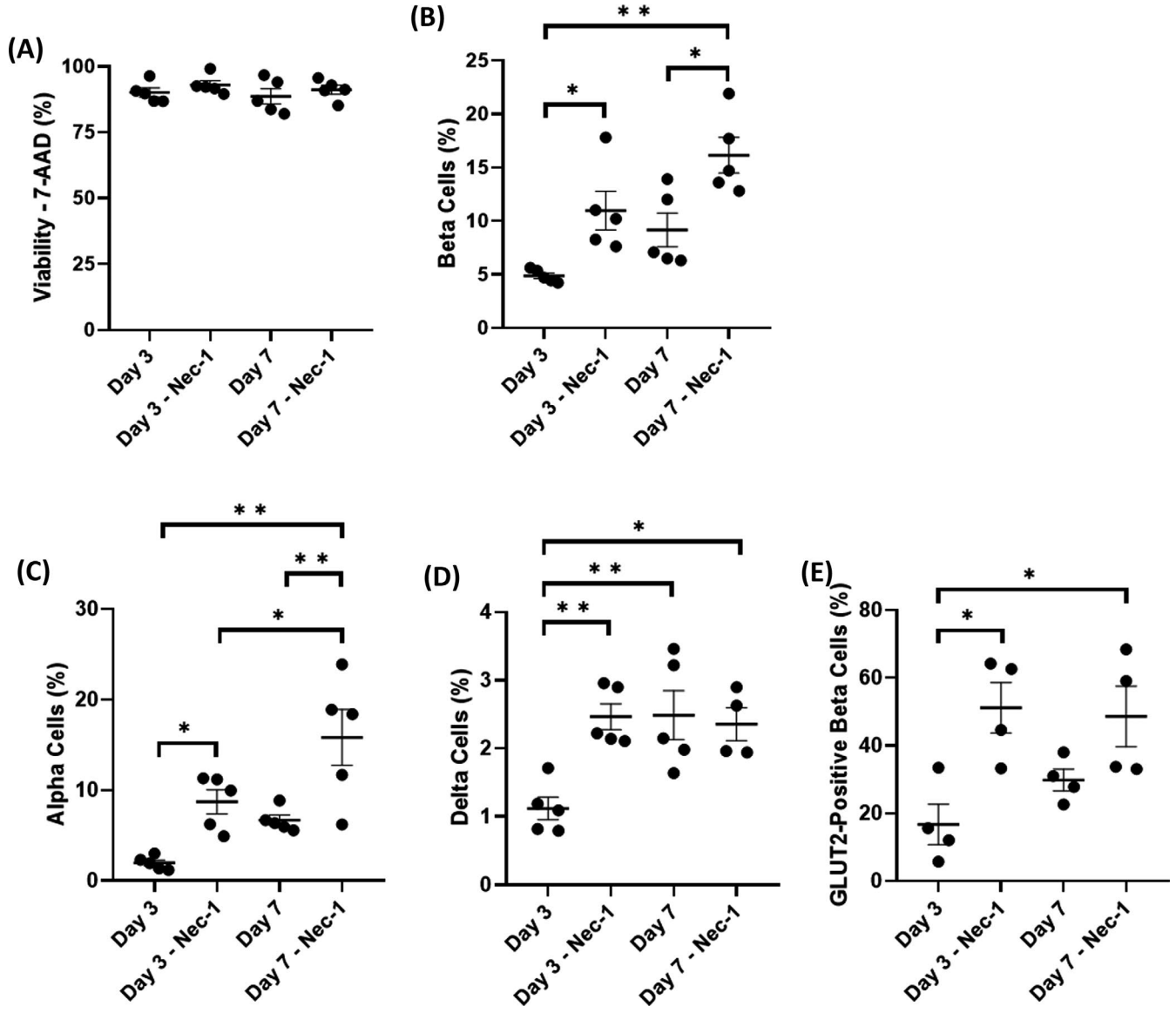

FIGURE 4 Flow cytometric analysis of viability, cellular composition, and GLUT2 expression in beta cells of PPIs on days 3 and 7 in control media or media supplemented with Nec-1 on day 0 of culture. Islets were dissociated on days 3 and 7 of culture using Accutase, stained with 7-AAD viability dye, anti-insulin, anti-glucagon, anti-somatostatin, and anti-GLUT2 antibodies, and analyzed by flow cytometry. (A) The viability percentage of PPIs on days 3 and 7 of culture (n = 5 for each group). (B) The percentage of beta cells on days 3 and 7 of culture (n = 5 for each group). (C) The percentage of alpha cells on days 3 and 7 of culture (n = 5 for each group). (D) The percentage of delta cells on days 3 and 7 of culture (n = 5 for each group). (E) The percentage of GLUT-2-positive beta cells on days 3 and 7 of culture (n = 4 for each group). *P < .05. **P < .01. Data expressed as mean ± SEM

(L1 = 0.35 ± 0.05 pg/ng DNA/h, H = 0.57 ± 0.07 pg/ng DNA/h, L2 = 0.43 ± 0.04 pg/ng DNA/h, H+ = 1.12 ± 0.2 pg/ng DNA/h) (L1, L2, and H+: P < .01, H: P = NS, Figure 6A). Similarly, the amount of secreted insulin in L1, L2, and H+ but not H glucose media of Nec-1-treated islets on day 3 of culture was significantly higher than untreated islets on day 7 of culture (L1 = 0.39 ± 0.07 pg/ng DNA/h, H = 0.85 ± 0.13 pg/ng DNA/h, L2 = 0.60 ± 0.08 pg/ng DNA/h, H+ = 1.22 ± 0.29 pg/ng DNA/h) (L1, L2, and H+: P < .01, H: P = NS, Figure 6A).

When compared to untreated islets on day 3 of culture, Nec-1-treated islets on day 7 of culture (L1 = 2.90 ± 0.42 pg/ng DNA/h, H = 6.85 ± 1.15 pg/ng DNA/h, L2 = 2.24 ± 0.30 pg/ng DNA/h, H+ = 5.99 ± 1.28 pg/ng DNA/h) had a 5-fold increase in L1 (P < .01), H (P < .01), L2 (P < .01), and H+ (P < .01) glucose media (L1, H, L2, and H+: P < .01, Figure 6A). On day 7 of culture, Nec-1 treatment increases the amount of secreted insulin from islets in L1, H, L2, and H+ glucose media by 7-fold, 8-fold, 3-fold, and 5-fold, respectively (L1, H, L2, and H+: P < .01, Figure 6A).

While prolonged culture from day 3 to day 7 did not improve the amount of insulin secreted in the untreated group for all glucose media concentration, Nec-1 treatment significantly improved the insulin secretion from islets on day 7 compared to day 3 in L1,

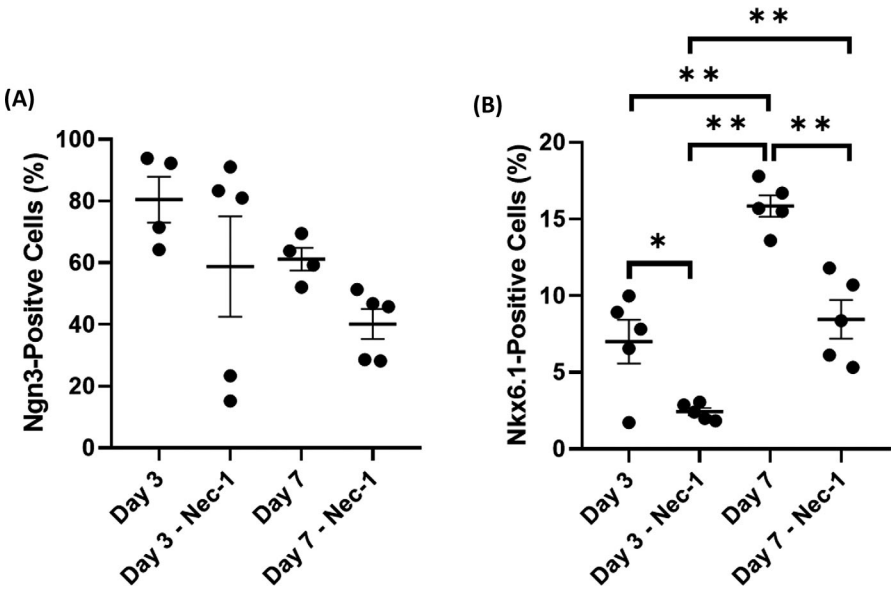

FIGURE 5 Flow cytometric analysis of endocrine cell differentiation of PPIs on days 3 and 7 in control media or media supplemented with Nec-1 on day 0 of culture. Islets were dissociated on days 3 and 7 of culture using Accutase, stained with 7-AAD viability dye, anti-Ngn3 and anti-Nkx6.1 antibodies, and analyzed by flow cytometry. (A) The percentage of Ngn3-positive cells on days 3 and 7 of culture (n = 4 for untreated groups, n = 5 for Nec-1-treated groups). (B) The percentage of Nkx6.1-positive cells on days 3 and 7 of culture (n = 5 for each group). *P < .05. **P < .01. Data expressed as mean ± SEM

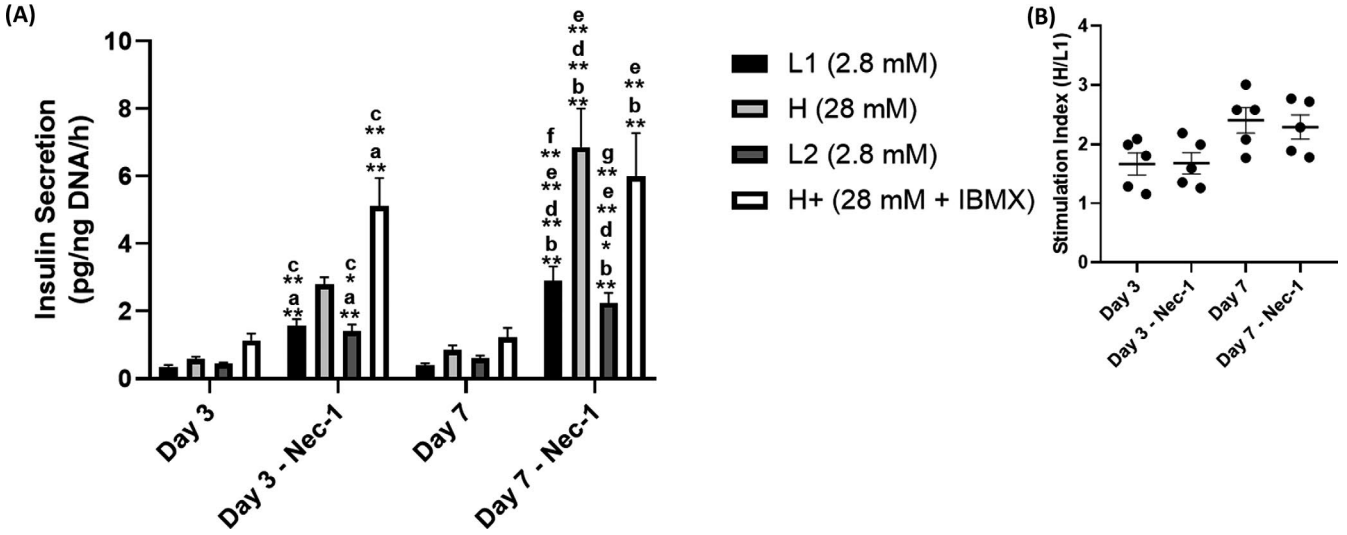

FIGURE 6 Function of PPIs in response to glucose challenge on days 3 and 7 of culture in control media or media supplemented with Nec-1 on day 0 of culture. Islet function was evaluated using glucose-stimulated insulin release assay. A triplicate of 100 IEQ was incubated for 1 hour in media with the corresponding order of glucose concentration: 2.8 mM (L1), 28 mM (H) 2.8 mM (L2), and 28 mM + 0.1 mM IBMX (H+), glucose media. The concentration of secreted insulin from each media condition was quantified by ELISA and normalized to the sample DNA content. (A) Insulin secretion per ng DNA after incubation in varying concentration of glucose media released by PPIs on days 3 and 7 (n = 5 for each group). (B) Stimulation index, calculated as the insulin secretion in H media over L1 media, of PPIs on days 3 and 7 (n = 5 for each group). *P < .05. **P < .01. Data expressed as mean ± SEM. a. Day 3 vs Day 3 – Nec-1; b. Day 3 vs Day 7 – Nec-1; c. Day 3 – Nec-1 vs Day 7; d. Day 3 – Nec-1 vs Day 7 – Nec-1; e. Day 7 vs Day 7 – Nec-1; f. Day 7 – Nec-1 – L1 vs Day 7 – Nec-1 – H; g. Day 7 – Nec-1 – L2 vs Day 7 – Nec-1 – H

H, and L2 glucose media (L1 and H: P < .01, L2: P < .05, Figure 6A). Additionally, only Nec-1-treated islets on day 7 of culture had a significantly higher insulin secretion in H glucose media compared to L1 and L2 glucose media (P < .01, Figure 6A).

Even though longer culture time improved the stimulation index, there were no statistically significant differences in the stimulation indices between untreated (day 3 = 1.67 ± 0.19 and day 7 = 2.40 ± 0.22) and Nec-1-treated islets (day 3 = 1.68 ± 0.18 and day 7 = 2.29 ± 0.20) on both day 3 and 7 of culture (P = NS, Figure 6B).

3.7 | The effect of Nec-1 supplementation to islet culture media on islet oxygen consumption rate

The metabolic potency of untreated and Nec-1-treated islets was evaluated by measuring the oxygen consumption rate on day 3 and day 7 of culture and normalized to the total DNA. The OCR/DNA of untreated (day 3 = 384.41 ± 27.14 nmol/min•mg DNA and day 7 = 338.83 ± 51.58 nmol/min•mg DNA) and Nec-1-treated islets (day 3 = 378.72 ± 56.46 nmol/min•mg DNA and day

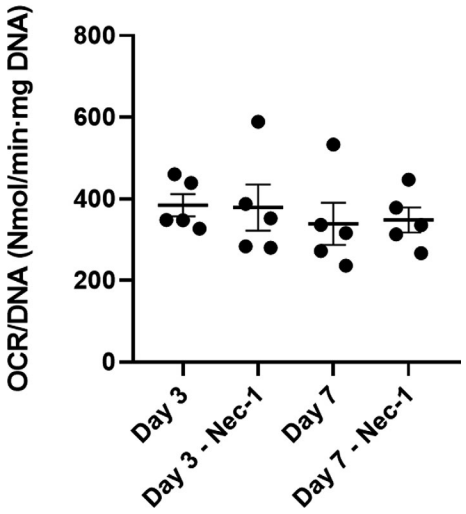

FIGURE 7 Oxygen consumption rate of PPIs on days 3 and 7 of culture in control media or media supplemented with Nec-1 on day 0 of culture. 200 IEQ per isolation was evaluated for metabolic potency on days 3 and 7 using a fiber optic sensor monitoring system and expressed as oxygen consumption rate normalized to the total DNA (n = 5 for each group). Data expressed as mean ± SEM

7 = 348.20 ± 30.52 nmol/min•mg DNA) were similar throughout 7-day culture (*P* = NS, Figure 7).

4 | DISCUSSION

Pre-transplant culture is essential for the maturation of young porcine islets after isolation.[12,14-17] This prolonged culture is associated with marked islet loss.[14,17] Therefore, the identification of cytoprotective agents to improve islet quantity and quality during pre-transplant culture before islet transplantation is critical to optimize the current islet transplantation protocol. Nec-1, a potent inhibitor of necroptosis, has the potential to improve islet survival during in vitro maturation culture. Here, we present a comprehensive analysis of the effects of Nec-1 supplementation to culture media after islet isolation. We examined the recovery, differentiation, GLUT2 expression, and insulin secretory function of pre-weaned porcine islets after 7-day culture. Surprisingly, the addition of Nec-1 to culture media did not improve parameters to assess islet survival after 7-day culture, such as recovery, viability, and oxygen consumption. However, Nec-1 significantly increased the islet insulin content, endocrine cellular composition, GLUT2 expression, and insulin response to glucose challenge.

Our findings that Nec-1 treatment did not prevent islet loss throughout 7-day culture supports previously published results that encapsulated human islets with Nec-1 did not significantly improve cell survival in either normal or low-nutrient condition at normal oxygen level.[29] The decrease in porcine islet recovery throughout culture in this study is consistent with a previous study, which demonstrated a 51% and 72% reduction in the neonatal porcine islet equivalent on day 12 and day 27 of culture, respectively.[17] Nec-1 supplementation did not result in a significant improvement in islet viability, possibly due to the high viability in both untreated and Nec-1-treated group (>85% viable, on average). These results support previous findings that young porcine maintain an average viability of 80% throughout 27-day culture.[12,14,17] The 2-fold increase in insulin content after 7-day treatment with Nec-1 represents a substantial benefit as transplanting a similar islet equivalent of Nec-1-treated islets would result in an islet graft with significantly higher insulin content. While the effects of Nec-1 to improve cell survival have been extensively studied, our novel finding that Nec-1 treatment could improve islet insulin content, to our best knowledge, has not been documented in the literature. Nec-1 exhibits its action by blocking the RIP1 kinase in the necroptosis signaling cascade to prevent plasma membrane rupture.[36] The mechanism by which Nec-1 or the inhibition of RIP1 kinase could increase islet insulin content remains to be investigated.

The increase in islet insulin content could be attributed to the significant expansion of beta-cell composition on both days 3 and 7 of culture after Nec-1 treatment. While a previous study has demonstrated that culturing young porcine islets for 20 days in a multistep differentiation media involving the use of 6 different agents, including oncostatin M, dexamethasone, nicotinamide, exendin-4, TGF-ß1, and thrombin, resulted in a 2-fold increase in the composition of beta and alpha cells, Nec-1 treatment alone in our study increased the proportion of beta, alpha, and delta cells by 2-fold on both days 3 and 7 of culture.[16] The transcription factor Ngn3 directs the differentiation of pancreatic precursor cells toward the development of endocrine cells, but is suppressed after final differentiation of endocrine cells.[37-39] In accordance with the increase in the composition of mature endocrine cells with prolonged culture in both control and untreated islets, the level of Ngn3-positive endocrine progenitor cells decreased with culture time in our study, indicating the development of mature endocrine cells from progenitor cells. This result supports other published findings that the expression of Ngn3 in human embryonic stem cells was downregulated as the expression of mature endocrine cells, insulin, glucagon, and somatostatin increased after 24-day differentiation culture. On average, the level of Ngn3-positive endocrine progenitor cells was lower in Nec-1-treated islets compared to untreated islets on days 3 and 7 of culture, which further supports our findings that Nec-1 increased the content of mature endocrine cells on both day 3 and day 7 of culture. The NK homeobox factor Nkx6.1 is crucial for endocrine progenitor cells that have progressed past the Ngn3 differentiation stage to develop into beta cells.[41,42] Consistent with a published report by Cai et al,[40] this study found that Nkx6.1-positive progenitor cells significantly increased from day 3 to day 7 of culture, supporting the in vitro differentiation of beta cells in maturation culture media. Our findings that Nec-1-treated islets had significantly lower Nkx6.1-positive cells but significantly higher beta cells on both day 3 and day 7 of culture compared with untreated islets indicate that Nec-1 treatment may accelerate the differentiation of Nkx6.1-positive progenitor cells into mature beta cells. A recent study showed that upregulation of the transcription factor Pdx-1 stimulated both

beta- and alpha-cell proliferation through a distinct pathway from Nkx6.1-stimulated proliferation.[43] Due to the increase in both beta- and alpha-cell contents after Nec-1 treatment, it is possible that Nec-1 treatment upregulated PDX-1 or its downstream targets to stimulate beta- and alpha-cell proliferation. As there exists an insulin-negative/Nkx6.1-positive cell population that actively replicates during pancreatic endocrine cell development, the higher content of Nkx6.1-positive cells in the untreated group could indicate a population of replicating insulin-negative/Nkx6.1-positive progenitor cells for differentiation into insulin-positive beta cells.[41] While the effect of Nec-1 on islet development has not been demonstrated, our findings that Nec-1 treatment enhanced endocrine cell expansion during culture warrant further studies into the novel mechanism of Nec-1.

While young porcine islets can serve as an unlimited source of islet, the delayed function requiring prolonged maturation culture hinders their widespread application. The glucose transporter GLUT2 is responsible for glucose sensing and glucose uptake in beta cells.[44] GLUT2 has been shown to be highly expressed in porcine pancreatic islets. Recent findings have reported that the GLUT2 expression of neonatal porcine islets was 2-fold lower than adult pig islets, which correlated to a significant reduction in glucose-stimulated insulin release.[13] Another study demonstrated that the increased insulin output in the presence of superoxide dismutase was correlated to enhanced GLUT2 expression in porcine islets.[45] While prolonged culture did not increase GLUT2 expression, treatment of pre-weaned porcine islets with Nec-1 in our study led to a 2-fold increase in GLUT2 expression of beta cells on both day 3 and day 7 of culture. In addition to the improved insulin content, the enhanced differentiation of beta cells and expression of GLUT2 in pre-weaned porcine islets after Nec-1 treatment likely accounted for the significant increase in insulin secretion during glucose challenge. The delayed function of immature young porcine islets could potentially be due to the substantially lower absolute amount of insulin secreted in response to glucose challenge compared to adult porcine islets.[13] After Nec-1 treatment, pre-weaned porcine islets son day 7 of culture had comparable absolute amount of insulin secreted normalized to cellular DNA during both basal and stimulated glucose conditions to adult porcine islets.[13] In addition, Nec-1-treated islets on day 7 of culture had a 2-fold increase in the amount of insulin secreted when exposed to 2.8 mM glucose media in comparison with adult porcine islets.[13] Notably, only Nec-1-treated islets on day 7 of culture had a significantly lower amount of insulin secreted when exposed to 2.8 mM glucose media before and after incubation in 28 mM glucose media, indicating a return to basal insulin secretion and improved response to glucose challenge. Human islets with an OCR/DNA of greater than 125 nmol/min•mg DNA had a significantly higher diabetes reversal rate in nude mice compared to those with lower OCR/DNA.[46] When compared to untreated islets on both day 3 and day 7 of culture, the non-significant difference in the OCR/DNA of Nec-1-treated islets could be due to the high OCR/DNA (>300 nmol/min•mg DNA, on average) in both groups. These results support previous findings that oxygen consumption rate was not correlated to either static or dynamic glucose-stimulated insulin secretion in both adult and young porcine islets.[13] As a higher OCR/DNA has been correlated to improved diabetes reversal in adult porcine islets, the high OCR/DNA and increased insulin secretion in pre-weaned porcine islets after Nec-1 treatment support further studies to determine the in vivo function of Nec-1-treated islets.[47]

There are certain limitations to our study. The mechanism by which Nec-1 enhanced the expansion of pancreatic endocrine cells was not fully explored. Our findings that Nec-1 acted on key transcription factors, Ngn3 and Nkx6.1, to promote the development of endocrine cells in pancreatic islets suggest further studies to explore the novel effects of Nec-1 on signaling molecules of the pancreatic endocrine maturation pathways. The function of Nec-1-treated islets was only assessed using in vitro glucose-stimulated insulin secretion assay. Future studies will need to be conducted to determine whether the increased insulin content and insulin secretion can translate into improved in vivo function and better diabetes reversal.

5 | CONCLUSION

To our best knowledge, this is the first study to present the novel effect of Nec-1 to improve maturation and enhance insulin secretory function of pre-weaned porcine islets during 7-day culture. Nec-1 treatment significantly increased islet insulin content, augmented insulin secretion in response to glucose challenge, upregulated GLUT2 expression, and facilitated the development of mature endocrine cells. Future studies will be conducted to determine the optimal dose and time course of Nec-1 as well as evaluate the in vivo function of Nec-1-treated islets in animal models of human type 1 diabetes. As young porcine islets represent a promising alternative source of islets but are functionally immature, Nec-1 supplementation to maturation culture prior to transplantation will enhance islet quality and assist to advance clinical islet xenotransplantation.

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REFERENCES

1. Shapiro AM, Lakey JR, Ryan EA, et al. Islet transplantation in seven patients with type 1 diabetes mellitus using a glucocorticoid-free immunosuppressive regimen. *N Engl J Med*. 2000;343(4):230-238.
2. Matsumoto S, Noguchi H, Naziruddin B, et al. Improvement of pancreatic islet cell isolation for transplantation. *Proceedings*. 2007;20(4):357-362.
3. Robertson RP. Islet transplantation as a treatment for diabetes – a work in progress. *N Engl J Med*. 2004;350(7):694-705.
4. CITR Coordinating Center. 10th annual report. CITR Collaborative Islet Transplant Registry. http://www.citregistry.org/. Web site. Updated 2017. Accessed April 30, 2019.
5. Boker A, Rothenberg L, Hernandez C, Kenyon NS, Ricordi C, Alejandro R. Human islet transplantation: update. *World J Surg*. 2001;25(4):481-486.
6. Ekser B, Bottino R, Cooper D. Clinical islet xenotransplantation: a step forward. *EBioMedicine*. 2016;12:22-23.
7. Yang L, Guell M, Niu D, et al. Genome-wide inactivation of porcine endogenous retroviruses (PERVs). *Science*. 2015;350(6264):1101-1104.
8. Lai L, Kolber-Simonds D, Park KW, et al. Production of alpha-1,3-galactosyltransferase knockout pigs by nuclear transfer cloning. *Science*. 2002;295(5557):1089-1092.
9. Phelps CJ, Koike C, Vaught TD, et al. Production of alpha 1,3-galactosyltransferase-deficient pigs. *Science*. 2003;299(5605):411-414.
10. Kemter E, Wolf E. Recent progress in porcine islet isolation, culture and engraftment strategies for xenotransplantation. *Curr Opin Organ Transplant*. 2018;23(6):633-641.
11. van der Windt DJ, Bottino R, Kumar G, et al. Clinical islet xenotransplantation: how close are we? *Diabetes*. 2012;61(12):3046-3055.
12. Lau H, Corrales N, Lee S, et al. Exendin-4 improves yield and function of isolated pre- weaned porcine islets. *J Endocrinol Diabetes*. 2018;5(5):1-7.
13. Smith KE, Purvis WG, Davis MA, et al. In vitro characterization of neonatal, juvenile, and adult porcine islet oxygen demand, beta-cell function, and transcriptomes. *Xenotransplantation*. 2018;25(6):e12432.
14. Lamb M, Laugenour K, Liang O, Alexander M, Foster CE, Lakey JR. In vitro maturation of viable islets from partially digested young pig pancreas. *Cell Transplant*. 2014;23(3):263-272.
15. Ellis C, Lyon JG, Korbutt GS. Optimization and scale-up isolation and culture of neonatal porcine islets: potential for clinical application. *Cell Transplant*. 2016;25(3):539-547.
16. Hassouna T, Seeberger KL, Salama B, Korbutt GS. Functional maturation and in vitro differentiation of neonatal porcine islet grafts. *Transplantation*. 2018;102(10):e413-e423.
17. Jimenez-Vera E, Davies S, Phillips P, O'Connell PJ, Hawthorne WJ. Long-term cultured neonatal islet cell clusters demonstrate better outcomes for reversal of diabetes: in vivo and molecular profiles. *Xenotransplantation*. 2015;22(2):114-123.
18. Kin T, Senior P, O'Gorman D, Richer B, Salam A, Shapiro AM. Risk factors for islet loss during culture prior to transplantation. *Transpl Int*. 2008;21(11):1029-1035.
19. Bruni A, Bornstein S, Linkermann A, Shapiro A. Regulated cell death seen through the lens of islet transplantation. *Cell Transpl*. 2018;27(6):890-901.
20. Rojas J, Bermudez V, Palmar J, et al. Pancreatic beta cell death: novel potential mechanisms in diabetes therapy. *J Diabetes Res*. 2018;2018(9601801):1-19.
21. Itoh T, Takita M, SoRelle JA, et al. Correlation of released HMGB1 levels with the degree of islet damage in mice and humans and with the outcomes of islet transplantation in mice. *Cell Transplant*. 2012;21(7):1371-1381.
22. Itoh T, Iwahashi S, Kanak MA, et al. Elevation of high-mobility group box 1 after clinical autologous islet transplantation and its inverse correlation with outcomes. *Cell Transplant*. 2014;23(2):153-165.
23. Tamura Y, Chiba Y, Tanioka T, et al. NO donor induces Nec-1-inhibitable, but RIP1-independent, necrotic cell death in pancreatic β-cells. *FEBS Lett*. 2011;585(19):3058-3064.
24. Degterev A, Hitomi J, Germscheid M, et al. Identification of RIP1 kinase as a specific cellular target of necrostatins. *Nat Chem Biol*. 2008;4(5):313-321.
25. Degterev A, Huang Z, Boyce M, et al. Chemical inhibitor of nonapoptotic cell death with therapeutic potential for ischemic brain injury. *Nat Chem Biol*. 2005;1(2):112-119.
26. Rosenbaum DM, Degterev A, David J, et al. Necroptosis, a novel form of caspase-independent cell death, contributes to neuronal damage in a retinal ischemia-reperfusion injury model. *J Neurosci Res*. 2010;88(7):1569-1576.
27. Lim SY, Davidson SM, Mocanu MM, Yellon DM, Smith CC. The cardioprotective effect of necrostatin requires the cyclophilin-D component of the mitochondrial permeability transition pore. *Cardiovasc Drugs Ther*. 2007;21(6):467-469.
28. Smith CC, Davidson SM, Lim SY, Simpkin JC, Hothersall JS, Yellon DM. Necrostatin: a potentially novel cardioprotective agent? *Cardiovasc Drugs Ther*. 2007;21(4):227-233.
29. Paredes-Juarez GA, Sahasrabudhe NM, Tjoelker RS, et al. DAMP production by human islets under low oxygen and nutrients in the presence or absence of an immunoisolating-capsule and necrostatin-1. *Sci Rep*. 2015;5:14623.
30. Ichii H, Inverardi L, Pileggi A, et al. A novel method for the assessment of cellular composition and beta-cell viability in human islet preparations. *Am J Transplant*. 2005;5(7):1635-1645.
31. Ricordi C, Gray DW, Hering BJ, et al. Islet isolation assessment in man and large animals. *Acta Diabetol Lat*. 1990;27(3):185-195.
32. Korbutt GS, Mallett AG, Ao Z, Flashner M, Rajotte RV. Improved survival of microencapsulated islets during in vitro culture and enhanced metabolic function following transplantation. *Diabetologia*. 2004;47(10):1810-1818.
33. Papas KK, Pisania A, Wu H, Weir GC, Colton CK. A stirred microchamber for oxygen consumption rate measurements with pancreatic islets. *Biotechnol Bioeng*. 2007;98(5):1071-1082.
34. Qi M, Bilbao S, Forouhar E, Kandeel F, Al-Abdullah IH. Encompassing ATP, DNA, insulin, and protein content for quantification and assessment of human pancreatic islets. *Cell Tissue Bank*. 2018;19(1):77-85.
35. Chakrabarti SK, Mirmira RG. Transcription factors direct the development and function of pancreatic beta cells. *Trends Endocrinol Metab*. 2003;14(2):78-84.
36. Linkermann A, Green DR. Necroptosis. *N Engl J Med*. 2014;370(5):455-465.
37. Schwitzgebel VM, Scheel DW, Conners JR, et al. Expression of neurogenin3 reveals an islet cell precursor population in the pancreas. *Development*. 2000;127(16):3533-3542.
38. Gradwohl G, Dierich A, LeMeur M, Guillemot F. neurogenin3 is required for the development of the four endocrine cell lineages of the pancreas. *Proc Natl Acad Sci USA*. 2000;97(4):1607-1611.
39. Sheets TP, Park K-E, Park C-H, et al. Targeted mutation of NGN3 gene disrupts pancreatic endocrine cell development in pigs. *Sci Rep*. 2018;8(1):3582.
40. Cai Q, Bonfanti P, Sambathkumar R, et al. Prospectively isolated NGN3-expressing progenitors from human embryonic stem cells give rise to pancreatic endocrine cells. *Stem Cells Transl Med*. 2014;3(4):489-499.
41. Sander M, Sussel L, Conners J, et al. Homeobox gene Nkx6.1 lies downstream of Nkx2.2 in the major pathway of beta-cell formation in the pancreas. *Development*. 2000;127(24):5533-5540.

42. Rudnick A, Ling TY, Odagiri H, Rutter WJ, German MS. Pancreatic beta cells express a diverse set of homeobox genes. *Proc Natl Acad Sci USA*. 1994;91(25):12203.
43. Hayes HL, Moss LG, Schisler JC, et al. Pdx-1 activates islet alpha- and beta-cell proliferation via a mechanism regulated by transient receptor potential cation channels 3 and 6 and extracellular signal-regulated kinases 1 and 2. *Mol Cell Biol*. 2013;33(20):4017-4029.
44. Thorens B. GLUT2, glucose sensing and glucose homeostasis. *Diabetologia*. 2015;58(2):221-232.
45. Giovagnoli S, Luca G, Casaburi I, et al. Long-term delivery of superoxide dismutase and catalase entrapped in poly(lactide-co-glycolide) microspheres: in vitro effects on isolated neonatal porcine pancreatic cell clusters. *J Control Release*. 2005;107(1):65-77.
46. Papas KK, Colton CK, Nelson RA, et al. Human islet oxygen consumption rate and DNA measurements predict diabetes reversal in nude mice. *Am J Transplant*. 2007;7(3):707-713.
47. Pepper AR, Hasilo CP, Melling CW, et al. The islet size to oxygen consumption ratio reliably predicts reversal of diabetes posttransplant. *Cell Transplant*. 2012;21(12):2797-2804.

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